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On-line supercritical fluid extraction-supercritical fluid reaction-capillary gas chromatography analysis of the fatty acid composition of oilseeds *

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Summary. The versatility of supercritical fluid extraction (SFE) directly coupled with gas chromatography (GC) is further expanded by the addition of an on-line derivatization technique that is performed under supercritical fluid conditions. The integrated extraction and derivatization technique has been applied to the determination of the fatty acid composition of oilseeds. Triglycerides are extracted from seed samples using SC-CO_2 and transesterified to methyl esters, in situ, over a solid catalyst. Experimental conditions were selected such that the methyl esters are preferentially eluted from the catalyst. A linear capillary restrictor was used to deposit the effluent from the supercritical fluid extractor/reactor onto a retention gap precolumn in the GC. Applications of the on-line SFE-SFR-GC technique include the analysis of single soybean, evening primrose, and peanut seeds.

Introduction

Reactions in supercritical fluids have recently been utilized in the fields of process chemistry and chemical engineering. Many applications of supercritical fluid reactions (SFR) are cited in the literature, including citations in biomass liquefaction [1], polymerization [2], as well as the treatment of hazardous wastes [3]. Considerable potential also exists in analytical chemistry for applying such reactions as enzymatic conversions [4–6], heterogeneous catalysis [7], and photolytically-induced transformations [8] in the presence of supercritical fluid media. Particularly attractive for the analyst is the ability to perform selective extractions from sample matrices in tandem with a reaction in a non-toxic and non-flammable medium, such as supercritical carbon dioxide (SC-CO_2). The capability of selectively removing reaction products, accelerating reaction rates, and conducting reactions in the absence of oxygen further enhances the potential application of SFRs in analytical chemistry.

This study reports on the development and testing of a simple technique for determining the fatty acid compositions of lipid-containing matrices utilizing a SFR both off-line and on-line in conjunction with a SFE-GC system. Selective removal and transfer of the lipid fraction from the sample matrix to the derivatization zone is accomplished using SFE. Transesterification of the triglycerides to fatty acid methyl esters is performed over alumina in the presence of supercritical carbon dioxide. The resultant methyl esters can be collected by decompressing the SC-CO_2 into a collection vial, or in the on-line technique, onto a retention gap.

Recent studies have demonstrated that the direct coupling of supercritical fluid extraction with gas chromatography (SFE-GC) is a viable analytical method [9–16] that can provide rapid and accurate analyses. For example, SFE-GC analysis of polynuclear aromatic hydrocarbons in a standard reference sample of urban dust [13] yields quantitative results in excellent agreement with those obtained using conventional liquid solvent extraction in the analysis. In this particular technique, both extraction and concentration steps for the SFE-GC method required only 15 min. By contrast, the conventional method using a Soxhlet apparatus, requires 48 h for extraction and 3 h for the concentration step. To date, a wide variety of sample matrices and analytes have been successfully analyzed using on-column SFE-GC [17].

It would appear that the first reported use of SFR in chemical analysis was by Liebman et al. [18] who examined the decomposition of propellants in supercritical carbon dioxide. Stopped-flow studies on packed columns have also shown that chemical reactions can occur in supercritical carbon dioxide under conditions commonly used in SFC [19]. Fields and Grolimund [20, 21] have also reported on the reaction of amine solutes in SC-CO_2 during supercritical fluid chromatography (SFC). Recently several investigators have introduced traditional derivatization agents into SFE

* Dedicated to Prof. Dr. Ernst F. G. Klesper on the occasion of his 65th birthday

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cells to enhance the extraction of very polar analytes [22–24] from a variety of sample matrices. However the method reported here involves a discrete extraction step followed by a reaction zone, and hence has the potential for scale up into a production process.

Experimental

SFE-Apparatus

On-line SFE was performed with a syringe pump, (Model SFC-500 Microflow Pump, ISCO, Inc. Lincoln, NE, USA) controlled by a IBM PC/XT computer via an interface using associated software and a Chemresearch SFC-500 Pump Controller from ISCO, Inc. The pump was also equipped with a cooling jacket which was refrigerated using a recirculating bath (Model RTE-110, Neslab, Portsmouth, NH, USA). The carbon dioxide extraction fluid (SFC grade, Scott Specialty Gases, Inc., Plumsteadville, PA, USA) was supplied in a cylinder with a dip tube. An HPLC column oven (Bio-Rad, Inc., Richmond, CA, USA) was used to control the temperature on the extraction cell. The cell was constructed from stainless steel reducing unions (1/4 – 1/16 inch o.d.) incorporating stainless steel frits of two micron porosity (SS-400-6-1ZVS5, Swagelok, Solon, OH, USA) and a 70 mm × 4.1 mm i.d. stainless steel tubing as the body of the extraction cell. The flow rate of the supercritical carbon dioxide through the cell was controlled by an outlet restrictor made from 0.375 mm o.d., 0.025 mm i.d. fused silica tubing (Polymicro Technologies, Phoenix, AZ, USA) of 23 cm in length.

Off-line SFE was carried out using a Hewlett-Packard Model 1082 supercritical fluid chromatograph (Hewlett-Packard Company, Avondale, PA, USA) that was modified to operate as an SFE system. In this arrangement, the extraction cell replaced the chromatographic column. The exit line was redirected outside the oven, thereby bypassing the UV detector. A fused silica restrictor (70 mm × 0.375 mm o.d. × 0.032 mm i.d.) was attached to the exit line via a low dead-volume fitting. Extracted species were collected by placing the end of the restrictor into a graduated collection tube which contained up to 5 ml of n-hexane.

GC equipment

On-line SFE-SFR-GC experiments were performed using a Hewlett-Packard 5700A gas chromatograph equipped with a flame ionization detector. The retention gap, a deactivated 7 m × 0.53 mm i.d. fused silica capillary (J & W Scientific, Folsom, CA, USA), was connected to the head of the column using a press-fit connector (Supelco, Bellefonte, PA, USA). The column (SP2330, Supelco) was 30 m × 0.32 mm i.d. The other end of the retention gap was connected to a splitless inlet linear located in the GC injection port. The GC carrier gas was helium at a linear velocity of 30 cm/s.

During the extraction step, the oven was kept at room temperature. The SFE cell and GC were coupled by first removing the septum with its cap and inserting the restrictor through the on-column injector. The outlet end of the restrictor was placed about 5 cm into the retention gap. After completion of the extraction/collection step, the restrictor was removed from the GC inlet and the injection port capped with its septum. Helium was allowed to purge the column for 8 min before the oven was heated ballistically to 100° C and held there for 4 min. The oven temperature was then ramped at 8° C/min to 240° C and held there for the duration of the chromatography. Standard solutions of fatty acid methyl esters (FAMES) (Nu Chek Prep, Elysian, MN, USA) were used to identify peak retention times.

Off-line GC analysis was performed using a GC (Model 3700, Varian, San Fernando, CA, USA) equipped with an FID and a 30 m × 0.25 mm i.d. capillary column (SP2340, Supelco). The GC was operated isothermally at 190° C with a split ratio of 100/1. The methyl esters were confirmed by GC/MS (GC: Model 3400, Varian; MS: Incos 50, Finnigan, Cincinnati, OH, USA) using the electron ionization mode.

Supercritical fluid reaction

The SFR conversion uses a packed bed of alumina, that has been pretreated with methanol, as a catalyst to directly transesterify the triglycerides to fatty acid methyl esters. The reaction is performed under conditions that preferentially elute the fatty acid methyl esters. The extraction/reaction was conducted at 200 bar and 60° C using approximately 1.3 g of alumina pretreated with methanol. The alumina (Neutral-Brockmann Activity I, 80 – 200 mesh, Fisher Scientific, Fairlawn, NJ, USA) was pretreated with a 8% (v/v{liquid}) $MeOH/CO_2$ stream at 60° C for 40 min at 1.0 ml/min liquid flow rate. The methanol/carbon dioxide mixture was then purged from the packed alumina bed with pure carbon dioxide at the same conditions for 20 min.

Sample preparation

Conventional preparation of soybean seeds involved Soxhlet extraction using hexane followed by removal of the solvent and then degumming of the phospholipids in the oil [25]. Methyl esters were prepared from the triglycerides using sodium methoxide. Seeds of the evening primrose (*Oenothera biennis*), peanut (*Arachis hypogaea*), and soybean (*Glycine max*) were available in our laboratory. Seed coats of the peanut and soybean samples were removed before crushing the seed body with the aid of a mortar and pestle. Crushed seed amounting to 8 – 12 mg was placed in the extraction cells.

Results and discussion

Off-line SFE-SFR

The method of choice for routine fatty acid analysis of lipids is derivatization of the fatty acids to their respective methyl esters followed by GC of the mixture [26]. The SFR-based technique employs the same method; however the derivatization is achieved over a solid reagent within the SFE cell. Initial evaluation of the SFR-based technique was accomplished using an off-line collection mode. Figure 1 compares the chromatograms obtained from SFE-SFR of comminuted evening primrose seed and crushed peanut meal. The difference in their fatty acid methyl ester (FAME) distributions is readily apparent. The FAME distributions were characteristic of the respective oilseed and attest to the potential usefulness of the SFE-SFR technique for fatty acid profiling.

Table 1 lists the percent distribution of the FAMES obtained by the SFE-SFR method compared to the literature values for evening primrose seeds [27]. The FAME distribution is in agreement with the range of compositions re-

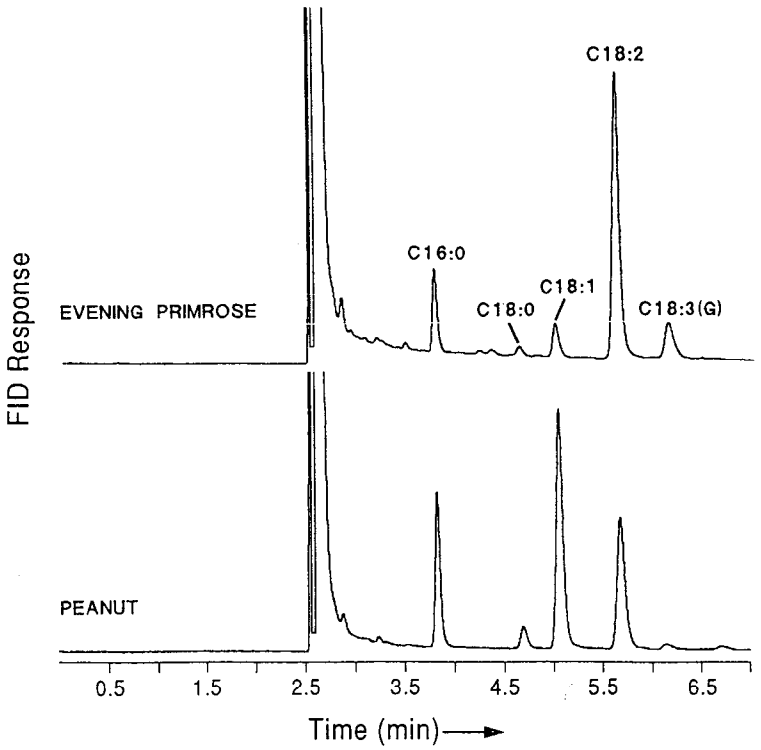

Fig. 1. GC-FID comparison of SFE-SFR on seed samples of evening primrose and peanut

ported for this oilseed type depending on its seasonal and geographic source. The fatty acid of therapeutic interest, gamma-linolenic acid, is clearly present and in the proportion normally observed in this seed type. FAME distributions were also successfully obtained for soybean flakes and a sample of tristearin. GC/MS was also used to confirm the identity of the methyl esters collected from the SFE/SFR of the soybean sample.

The above results clearly indicate that the transesterification of triglycerides with methanol is promoted under supercritical fluid conditions in the presence of alumina. Transesterification via heterogeneous catalysis has been demonstrated [28, 29] by several investigators, although not in the presence of supercritical carbon dioxide. The alcoholysis reaction [30] is normally a homogeneously catalyzed reaction that can be carried out quite easily at rather low temperatures (30 – 60° C), but requires extensive sample work-up to isolate the products after reaction. In the SFR technique, the methyl esters are directly formed and eluted from the alumina catalyst bed without the need for additional purification or separation. Fatty acids, diglycerides, and triglycerides are not eluted under the specified reaction or collection conditions. This was confirmed by analyzing the SFE/SFR effluent using supercritical fluid chromatography. Only peaks due to the methyl esters were observed in the chromatogram for the SFR fractions collected.

On-line SFE-SFR-GC

Figure 2 shows a simple schematic diagram of the combined SFE-SFR-GC system. The alumina is dry packed into the extraction cell allowing a small volume in the front of the cell to remain open to accommodate the seed sample. After pretreatment of the alumina bed with methanol, the cell is reopened and the sample placed at the head of the alumina column in the extraction cell.

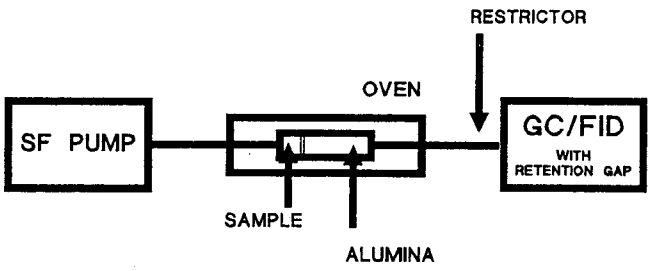

Fig. 2. Schematic diagram of on-line SFE-SFR-GC system

Table 1. Comparison of FAME distributions for *Oenothera biennis* (Evening Primrose) seeds

FAME	SFE-SFR method	Normal range [27]
16:0	12.4	7 – 10
18:0	1.7	1.5 – 3.5
18:1	7.4	6 – 11
18:2	68.0	65 – 80
18:3 (gamma)	10.6	8 – 14

On-line SFE-SFR-GC chromatograms of a soybean sample and sample blank are shown in Fig. 3. The blank run also contained the methanol pretreated alumina but no sample. No FAMES are evident in the blank run as shown and noted in Fig. 3. The broad solvent peak in the initial portion of the chromatogram is due to the co-extraction of

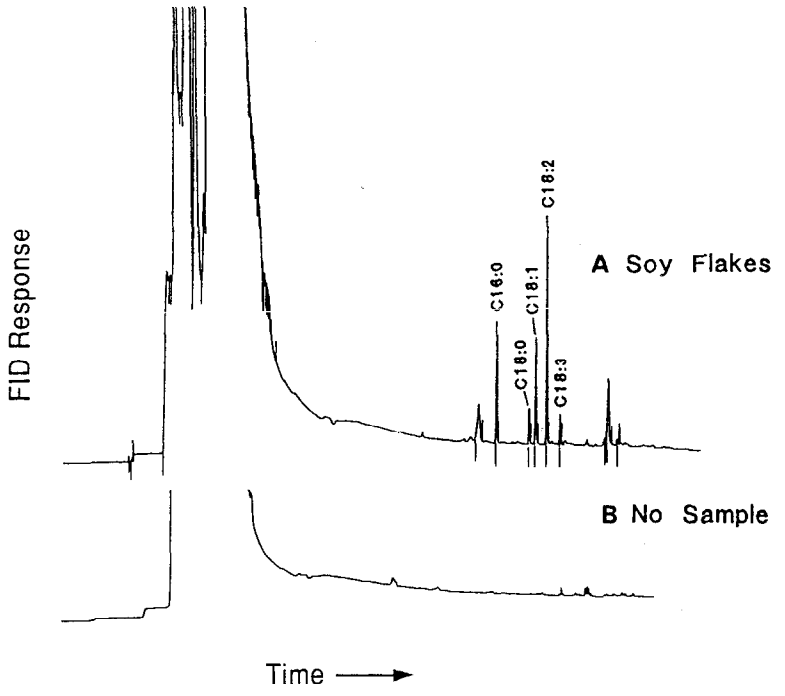

Fig. 3. On-line SFE-SFR-GC FAME analysis. (*A*) Soy flakes, (*B*) No sample

Table 2. Comparison of FAME distributions for *Glycine max.* (soybean)

FAME	On-line SFE-SFR-GC	Conventional method	Literature range
16:0	11.1	10.8	7–12
18:0	3.3	3.8	2– 5.5
18:1	25.3	22.9	20–50
18:2	52.2	54.5	35–60
18:3	8.0	8.1	2–13

Table 3. Comparison of steps for oilseed FAME analysis

SFE-SFR-GC	Conventional
SFE-SFR	Soxhlet extraction
GC analysis	Degumming
	Saponification
	Methylation
	Extraction
	Sample Cleanup
	GC analysis

some of the methanol from the alumina bed. This causes no difficulty in the chromatography, with the use of a retention gap, as detailed in the experimental section. The retention gap techniques [31] allows the solutes to be refocused onto the head of the capillary column, thereby eliminating the band proadening that would otherwise occur. The results shown in Fig. 3 clearly indicate the dissolution of the triglyceride-based seed oil in the SC-CO_2 prior to the SFR stage.

The FAME distribution obtained for the soybean sample by the on-line SFE-SFR-GC technique is listed in Table 2. The relative distribution is very similar to the one obtained by the conventional extraction and esterification method. Merely a portion of one soybean was used in the SFE-SFR-GC technique. Such small sample sizes make the technique amenable to plant breeding or genetic engineering studies, where it is common to have only a few seeds for analysis.

Another advantage of the SFE-SRC-GC techniques is that laboratory sample preparation protocol is substantially reduced. Table 3 tabulates the advantages of the SFE-SFR-GC technique versus the conventional methodology. Here the simplification of the FAME analysis by SFE-SFR-GC is readily evident. Major steps eliminated by the SFE-SFR technique are the Soxhlet extraction and solvent evaporation steps. In addition, no degumming step is required with the SFE-SFR techniques, since previous research in our laboratory has shown that phospholipids have negligible solubility in SC-CO_2 [32]. Again, the selectivity of the SFE-SFR technique eliminates any sample cleanup after the derivatization step, permitting direct interfacing to the GC.

Conclusions

The addition of supercritical fluid reaction step to an on-line SFE-GC method allows for the in-situ extraction, saponification, and alcoholysis-derivatization reaction analysis of the fatty acid composition of seed oils. The above-cited reaction is but one of many possibilities for integrating selective reaction chemistry conducting in supercritical fluid media into analytical methodology. Future research will focus on testing other catalysts and reaction conditions for accelerating the reaction rates for interestercation, including the analytical use of supported enzyme catalysts.

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